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14. ABSTRACT
The development of advanced prostate cancer (APC) after androgen deprivation therapy is characterized by reactivation of androgen receptor (AR) signaling within the tumor despite castrate levels of circulating androgens. This is caused by elevated intratumoral androgen biosynthesis and/or aberrant androgen receptor activation. Targeting both mechanisms of recurrence can provide superior therapeutic benefit relative to targeting either mechanism alone. AIdo-keto reductase 1C3 (AKR1C3) is highly upregulated in APC and is localized within the tumor. We report continuing pharmacological characterization of BMT 4-158, a bifunctional AKR1C3 inhibitor and AR antagonist. BMT4-158 competes with androgens for binding to the AR ligand binding domain (LBD) leading to reduced AR activation. It also blocks the androgen induced nuclear translocation that is essential for AR signaling. BMT4-158 is active in cellular context of both wild type and mutated AR. It reduces AR levels and inhibits the androgen dependent gene expression of PSA in parental LNCaP (AKR1C3 null) cells and LNCaP cells stably expressing AKR1C3. Bifunctional compounds like BMT4-158 have the potential to be superior agents for treatment of APC and may also surmount resistance to existing therapeutic agents like Abiraterone (Abi) and MDV3100 that target a single pathway.

15. SUBJECT TERMS
AR nuclear translocation, Competitive AR binding, AR dependent gene expression

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Introduction

Prostate cancer (PC) is the second most common malignancy in US men and accounts for about 30,000 deaths annually.[1, 2] Fatality from prostate cancer is usually due to the development of the castration resistant metastatic form of the disease known as advanced prostate cancer (APC) or castrate resistant prostate cancer (CRPC). APC develops as a result of intratumoral androgen biosynthesis independent of the testes and/or aberrant androgen receptor (AR) signaling.[3-5] The success of Abiraterone (Abi) and MDV3100 in clinical trials strongly supports targeting the androgen axis to develop novel and effective therapies for APC.[6-12] Abi inhibits CYP17α-hydroxylase/17,20-lyase (CYP17), an enzyme involved in androgen biosynthesis whereas MDV3100 is an AR antagonist that also leads to AR degradation. However, Abi also interferes with glucocorticoid metabolism and requires co-administration of prednisone while MDV3100 has been associated with dose-limiting CNS seizures.[12],[13] Drug resistance has also been reported with both compounds indicating the need for better therapeutic agents. Combination therapy of Abi with MDV3100 has been proposed as a way to reduce resistance.[14, 15] Aldo-keto reductase IC3 (AKR1C3, type 5 17β hydroxysteroid dehydrogenase) catalyzes the 17-keto-reduction of the weak androgen precursors Δ^4-androstene-3,17-dione (Δ^4-AD) and 5α-androstane-3,17-dione (5α-Adione) to yield testosterone and 5α-dihydrotestosterone (DHT), respectively.[16] AKR1C3 is also among the most highly upregulated enzymes in APC.[17-19] Unlike Abi, AKR1C3 inhibitors act further downstream and are potentially superior since they do not interfere with glucocorticoid biosynthesis. As part of our AKR1C3 drug discovery efforts, I identified 3-((4-nitronaphthalen-1-yl)amino)benzoic acid (BMT 4-158), a "first-in-class", dual acting AKR1C3 inhibitor and AR antagonist. BMT 4-158 potently and selectively inhibited AKR1C3 over closely related AKR1C enzymes, antagonizes DHT mediated AR transcriptional activity and reduced cellular AR levels in the presence and absence of DHT.[20] Use of bifunctional compounds that inhibit both androgen synthesis and AR activation is a novel concept which holds much promise. However, it is critical to understand how this compound inhibits AR and the functional implication of using this compound in prostate cancer cell lines that express AKR1C3 plus AR or AR alone. Here, I report experiments I have done to elucidate the mechanism(s) of its AR antagonism and evaluate its therapeutic potential in various APC settings using appropriate cells lines.

Body

The **statement of work** included the Objectives 1 and 2:

<u>Objective 1: Elucidate the mechanism of the AR antagonist properties of BMT4-158 (Months 1-15)</u>

Task 1.1 Conduct radioligand binding assays using [^{3}H]-R1881 (Months 1-2)

1.2 Evaluate the effect of BMT4-158 on agonist induced AR N/C interaction using a luciferase assay (Months 2-5)

1.3 Measure effect of BMT4-158 on AR nuclear translocation, AR gene expression and proteosomal degradation (Months 6-15)

1.4. Measure interaction of BMT4-158 with constitutively active AR splice variants (AR-V7, AR^{v567es} and AR3) (Months 6-15)

Anticipated Milestone(s) Achieved: Determination of the mechanism(s) of the AR antagonist action of BMT4-158

Objective 2: Evaluate efficacy of BMT 4-158 to inhibit cell proliferation and gene expression in prostate cancer cell lines (Months 16-24)

Task 2.1 Generate VCaP-shRNa-AKR1C3 cells (Months 16-17)
2.2 Determine the effect of BMT 4-158 on proliferation of cells with different AR and AKR1C3 background (Months 17-21)
2.3 Determine effect of BMT4-158 on basal and androgen induced expression of the AR dependent genes (PSA, TMPRSS2 and FASN) (Months 22-24)

Anticipated Milestone (s) Achieved: Characterization of effects of BMT4-158 on prostate cancer cell proliferation and AR dependent gene expression; publication of 2 peer reviewed papers

Progress on Objectives

The following specific aims were fully or partially completed within the period of time during which the postdoctoral fellowship was active.

A. Objective 1: Elucidate the mechanism of the AR antagonist properties of BMT4-158

Task 1.1: Conduct radioligand binding assays using [^{3}H]-R1881: A cell based radioliogand binding assay was established and the conditions optimized in place of the proposed radioligand binding assay. This assay was conducted using HeLa cells stably expressing the human wild type AR and PSA- luciferase reporter gene (HeLa13 cells)[21]. R1881, Bicalutamide and MDV3100 were used as controls

Task 1.3: Measure effect of BMT4-158 on AR nuclear translocation, AR gene expression and proteosomal degradation. HeLa13 cells were treated with the androgen 5α-dihydrotestosterone (DHT) in the presence and absence of BMT4-158 for 24h. The cell pellet was then fractionated into cytoplasmic and nuclear fraction. The fractions were analyzed by western blotting.

B. Objective 2: Evaluate efficacy of BMT 4-158 to inhibit cell proliferation and gene expression in prostate cancer cell lines.

Task 2.3: Determine effect of BMT4-158 on basal and androgen induced expression of the AR dependent genes (PSA, TMPRSS2 and FASN). LNCaP were treated with androgens in the presence and absence of BMT4-158 for 24h. LNCaP cells stably expressing AKR1C3 (LNCAP-AKR1C3 cells were likewise treated with either androgens or the AKR1C3 substrate, Δ^4-AD in the presence and absence of BMT4-158. Whole cells lysates obtained from the experiments were analyzed by western blotting and the level of prostate specific antigen (PSA) in the cells was used as a measure of androgen dependent gene expression.

Task 1.1: Conduct radioligand binding assays using [^{3}H]-R1881

Validation of the radioligand binding assay system was done by using cold R1881 to compete with 0.5 nM [^{3}H]-R1881 for binding to the AR. The IC_{50} value for cold R1881 was calculated to be 0.5 ± 0.04 nM. BMT4-158 inhibits the binding of [^{3}H]-R1881 to the AR in a concentration dependent manner with an IC_{50} value of 30 ± 5.1 µM. It was a weaker antiandrogen than bicalutamide (IC_{50} value = 430 ± 41 nM) and MDV3100 (IC_{50} value = 660 ± 100 nM). The ability of BMT4-158 to inhibit the binding of R1881 indicates that this compound inhibits AR signaling partly by binding to the ligand binding domain (LBD) of the androgen receptor to prevent agonist occupancy.

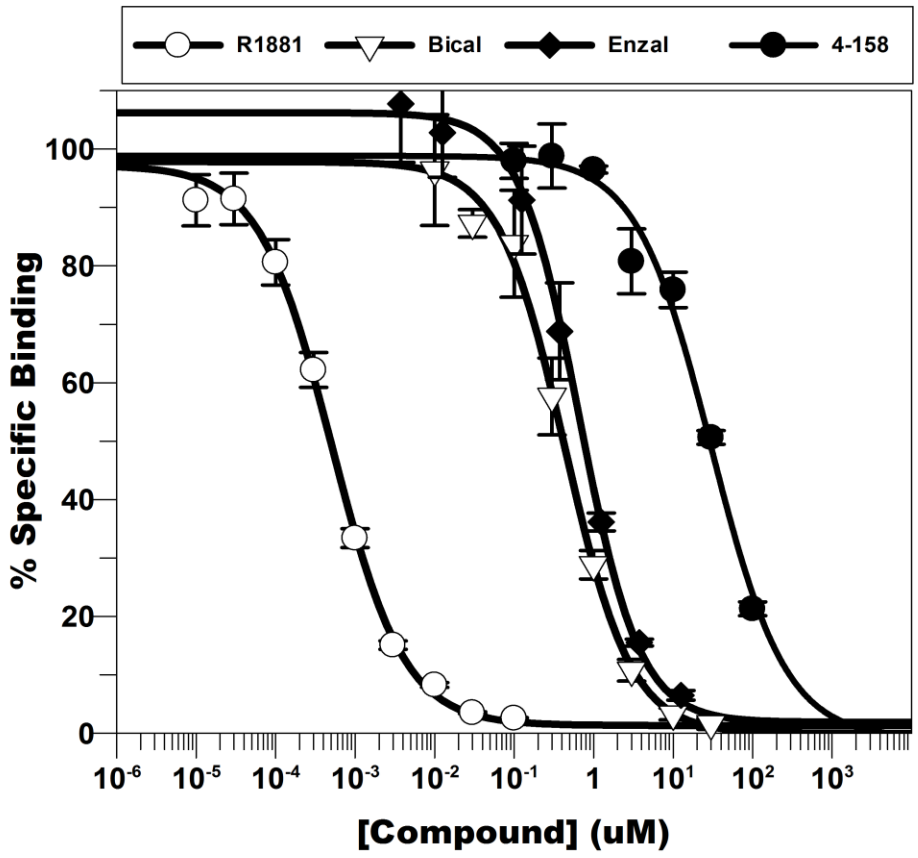

Figure 1: *BMT4-158 competes with [^{3}H]-R1881 for binding to the AR ligand binding domain in a competitive AR ligand binding assay in HeLa13 cells.*

To further gain insight into how the binding of BMT4-158 to the AR influences its ability to block AR signaling, a luciferase reporter gene assay in HeLa13 cells was conducted using the same concentration of the R1881 used in the binding assay. The potency of BMT4-158 in this assay, (IC_{50} value = 11 ± 2.4 µM) **Figure 2**, was three folds lower than the value obtained from the radioligand binding assay. This would suggest an additional mechanism of the action for this compound.

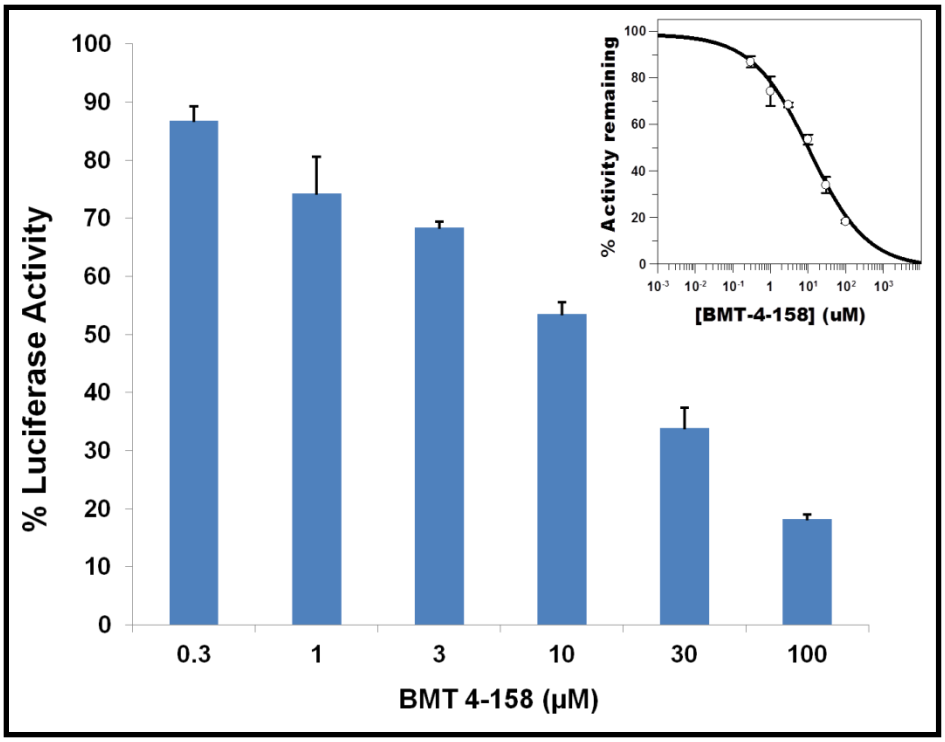

Figure 2: *BMT4-158 inhibits AR dependent luciferase reporter gene activity in HeLa13 cells when treated with 0.5 nM R1881.*

Task 1.3: Measure effect of BMT4-158 on AR nuclear translocation, AR gene expression and proteosomal degradation.

Treatment of HeLa13 cells with DHT led to significant translocation of the AR into the nucleus with little to no AR present in the nucleus after 24h whereas the AR was resident in the cytoplasm in untreated cells (**Figure 3**). BMT4-158 at low concentration (10 µM) did not have any effect on AR nuclear translocation following androgen treatment. However at higher concentration (40 µM) BMT4-158 was able to partially block the DHT dependent AR nuclear translocation. Interestingly, this response was similar to the effect of MDV3100 at 12.5 µM.

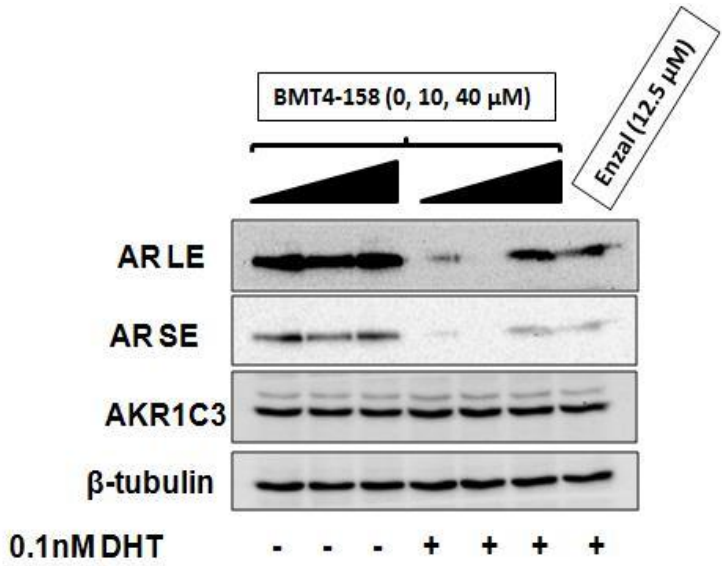

Figure 3*: Cytoplasmic fraction of HeLa13 cells. BMT4-158 partially blocks agonist induced AR nuclear translocation. (AR LE: AR- long exposure; AR SE: AR short exposure, Enzal: Enzaluatmide or MDV3100)*

From the results of the binding assay, MDV3100 completely prevents androgen binding at this concentration whereas there is still about 40% androgen binding at 40 µM BMT4-158. Ability of BMT 4-158 to block androgen dependent nuclear translocation therefore represents an additional mechanism by which this compound inhibits androgen signaling and might explain the disparity between the potency of BMT4-158 in the luciferase assay and the binding assay points to an additional mechanism of AR antagonism.

Task 2.3: Determine effect of BMT4-158 on basal and androgen induced expression of the AR dependent genes (PSA, TMPRSS2 and FASN).

Treatment of LNCaP cells with DHT led to an increase in expression of the androgen dependent gene PSA, **Figure 4A**. BMT4-158 was able to block the increase in DHT induced PSA expression at both concentrations of DHT used. Treatment of LNCaP cells with BMt4-158 also led to a decrease in the levels of AR within the cell, presumably by promoting the degradation of the AR. Since the LNCaP cells expressed the mutated AR (T877A), this observation shows the potential utility of this compound in advanced prostate cancer with a cellular context of both wild type and mutated AR.

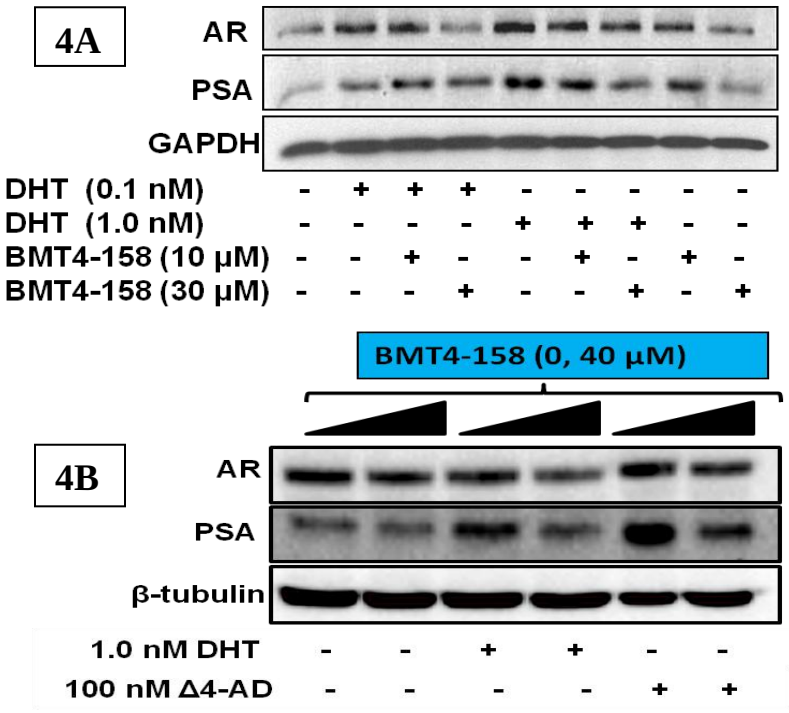

Figure 4. *BMT4-158 reduces cellular AR levels and blocks PSA expression in LNCaP cells (A) and in LNCaP-AKR1C3 cells treated with either DHT or the AKR1C3 substrate. Δ4-AD.*

Next LNCaP cells stably expressing AKR1C3 cells were treated with DHT and Δ4-AD in the presence and absence of BMT4-158 to evaluate the effect of BMT4-158 in a background of both AKR1C3 and AR. As shown in **Figure 4B,** treatment with DHT produces an increase in PSA expression that was inhibited by BMT4-158. Treatment with Δ4-AD produced a more robust increase in PSA expression that was likewise blocked by BMT4-158.

Key Research Accomplishments

- BMT4-158 competes with androgens for binding to the AR ligand binding domain and prevents AR signaling
- BMT4-158 inhibits nuclear translocation of the AR following androgen binding comparable to the effect seen with MDV3100
- BMT4-158 causes AR degradation and blocks AR dependent gene expression induced by androgens as well as the AKR1C3 substrate Δ*4-AD*
- BMT4-158 act as an antiandrogen against both wild type and mutated AR

Reportable Outcomes

Presentations:

1. Adeniji AO, Winkler JD, Penning TM. Pharmacological Characterization of a Bifunctional AKR1C3 Inhibitor and AR Antagonist. *Gordon Research Conference on Hormone Dependent Cancers (2013),* Smithfield, Rhode Island.

2. Adeniji AO. Development of a Novel Bifunctional AKR1C3 Inhibitor and AR Antagonist as a Therapeutic Agent for Prostate Cancer. *Biomedical Research Building (BRB II/III) 13th Floor Research In progress Meetings, University of Pennsylvania* (09/05/2013)

Conclusion

The goal of the project was to elucidate the mechanism of the antiandrogen effects of BMT4-158, a novel bifunctional aldo-keto reductase 1C3 inhibitor and androgen receptor antagonist, and to evaluate the efficacy of this compound in prostate cancer cells with different AR and AKR1C3 backgrounds. In this report, I show that BMT4-158 acts as an AR antagonist partly by binding to the AR-LBD thereby preventing agonist occupancy on the AR. It also precludes the nuclear translocation of the AR upon agonist binding, a critical step in AR signaling. BMT4-158 blocks transactivation of the AR by DHT (AR agonist) and Δ^4-AD (AKR1C3 substrate) prostate cancer cells. BMT4-158 is effective at degrading and blocking the activation of both wild type and mutated AR and blocks AR dependent gene expression in the context of either AR alone or AR and AKR1C3. This compound is a promising lead in the development of efficacious therapy for APC.

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Appendix

Personnel Supported by the Fellowship**:** Adegoke Adeniji PhD

Meeting Attended: Gordon Research Conference on Hormone Dependent Cancers, Smithfield, RI.

Date: 07/28/13- 08/02/13

Abstract:

The development of castration resistant prostate cancer (CRPC) after androgen deprivation therapy is characterized by reactivation of androgen receptor (AR) signaling within the tumor despite castrate levels of circulating androgens. This is caused by elevated intratumoral androgen biosynthesis and/or aberrant androgen receptor activation. Upregulation of key enzymes in the androgen biosynthetic pathways leads to increased androgen production while AR amplification and mutation contributes to the aberrant AR activation. Targeting both mechanisms can provide superior therapeutic benefit relative to targeting either mechanism alone. Aldo-keto reductase 1C3 (AKR1C3, type 5 17β-hydroxysteroid dehydrogenase) is highly upregulated in CRPC and is localized within the tumor. It catalyzes the formation of testosterone (T) and 5α-dihydrotestosterone (5α-DHT) from their inactive precursors. This makes AKR1C3 an appealing target for the treatment of CRPC. We report continuing pharmacological characterization of 3-((4-nitronaphthalen-1-yl)amino)benzoic acid (BMT 4-158), a first in class bifunctional agent that acts as an inhibitor of AKR1C3 and as an AR antagonist (*Bioorg Med Chem Lett* 2012, 22, 3492-3497). BMT4-158 competitively and selectively inhibits AKR1C3 in *in-vitro* assays using both steroidal (Δ^4-androstene-3,17-dione, Δ^4-AD) and non-steroidal ((*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol) substrates. BMT4-158 also blocks the conversion of Δ^4-AD to testosterone in LNCaP-AKR1C3 cells in a concentration dependent manner. The compound also inhibits the AR dependent luciferase activity with an EC value of 11 μM and leads to a reduction in AR levels within HeLa13 cells stably expressing the AR, in the presence and absence of DHT. It also reduces AR levels and inhibits the androgen dependent gene expression of PSA in parental LNCaP (AKR1C3 null) cells. Using two cells lines (HeLa13 and VCaP) that express the wild type AR, we show that BMT4-158 acts as an AR antagonist in part by its ability to prevent [^{3}H]-R1881 binding to the ligand binding domain (LBD) of the AR. Bifunctional compounds like BMT4-158 that inhibit both androgen biosynthesis and AR activation have the potential to be superior agents for treatment of CRPC and may also surmount resistance to existing therapeutic agents like Abiraterone (Abi) and Enzalutamide that target a single pathway.

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